DRUG DISCOVERY

Liver toxicity produced by the weight reducing agent 2,4 dinitrophenol

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ABSTRACT

Drug induced liver disease is an important and serious clinical problem. In this study, the effects of administering high doses of the sliming agent 2,4-DNP (2,4-DNP) on the rat liver was examined. Rats received intra peritoneal injections of 2,4-DNP (10, 40, 80, and 160 mg/kg) and euthanized after 4h. Measurements of malondialdehyde (lipid peroxidation) and reduced glutathione were done. Liver injury was evaluated by histopathology. Cell apoptosis was assessed by cleaved casapase-3 immunostaining. Masson's trichrome staining and liver hydroxyproline content were used to quantify collagenous fibers. Tissue content of mucopolysaccharides was assessed by the Periodic acid Schiff's (PAS) stain. Results showed that rats treated with 2,4-DNP exhibited significant inhibition of lipid peroxidation and marked decrease in reduced glutathione liver tissue levels. Severe liver injury occurred after 2,4-DNP indicated by liver inflammatory cell infiltrations and pyknotic hepatocytes progressing as the dose increased to massive degenerative changes, fibrosis and ultimately focal coagulative necrosis. There were also increased liver collagen and mucopolysaccharide contents. In addition, 2,4-DNP caused marked increments in cleaved caspase-3 expression in the liver. These results showed that high doses of the weight reducing agent 2,4-DNP can inflict serious liver injury as a consequence of uncoupling of oxidative phosphorylation and depletion of energy stores.

Keywords: Mitochondrial uncoupling, 2,4-dinitrophenol, weight loss, hepatic toxicity, apoptosis, necrosis

1. INTRODUCTION

2,4-DNP uncouples the electron transport chain from oxidative phosphorylation in mitochondria with the net result that the energy produced by redox reactions cannot be stored in the form of adenosine triphosphate (ATP) synthesis and is dissipated as heat (Terada, 1990). These effects of 2,4-DNP were the basis for its use as in the pharmacological treatment of obesity. Accordingly, the agent has been used in the 1930s to induce significant weight loss. Owing to the many fatalities from overdoses, it was banned in 1939 in the United States (Colman, 2007). 2,4-DNP, however, has regained popularity in the last decade as a weight reducing agent especially in body builders and is sold unregulated via the internet as a dietary supplement or chemical that reduces weight (Siegmueller

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and Narasimhaiah, 2010; Grundlingh et al., 2011). This coming back of 2,4-DNP was accompanied by an increasing number of reports describing toxicity and fatalities in those taking high doses of the agent. The common clinical features of these 2,4-DNP intoxications are uncontrollable hyperpyrexia, general unrest, haemodynamic instability, acute pulmonary oedema, respiratory failure, acute renal failure, metabolic acidosis, hyperkalaemia and cardiac arrest. There is no antidote for 2,4-DNP and the management of intoxication relies on supportive measures (Leftwich et al., 1982; Bartlett et al., 2010; Zack et al., 2016; Holborow et al., 2016).

The liver is the largest organ in the body which is the site for proteins, lipids and carbohydrate metabolism. It is also the major site where many xenobiotics and drugs are detoxified and eliminated. Some of these xenobiotics and chemicals and their metabolites can injure the liver causing degeneration and death of hepatocytes (Österreicher and Trauner, 2012). Many environmental chemicals exert their toxic action by interfering with mitochondrial bioenergetics. 2,4-DNP which causes ATP deficits and metabolic acidosis poses a high risk for the mitochondria and can result in catastrophic cell damage (Wallace and Starkov, 2000). In view of the above, we aimed in this study to investigate the effect of high doses of weight reducing agent 2,4-DNP on the liver tissue integrity using biochemical and histological measures.

2. MATERIALS AND METHODS

Animals

Female Sprague-Dawley strain rats weighing 120-130 g were used. Animals were group-housed under temperature- and light-controlled conditions and given standard laboratory rodent chow and tap water ad libitum. Animal procedures followed the guidelines of the Institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Chemicals and Reagents

2,4 dinitrophenol was purchased from Sigma (St. Louis, USA) and suspended in 1 w/v % methylcellulose solution. Other chemicals and reagents were of analytical grade and purchased from Sigma (St. Louis, USA).

Experimental Groups

Rats were randomly assigned into equal groups of 8 rats each. Group 1 was intraperitoneally treated with the vehicle and served as negative control. The other groups were treated with 2,4-DNP at doses of 10, 40, 80 and 160 mg/kg (i.p.) and euthanized by decapitation under light ether anesthesia after 4h, their livers were quickly removed. The left lobe of the liver from each animal of all groups were kept on ice-cold glass plate and stored at -80^{o}C for the biochemical assays. The right lobe of the liver from all dissected animals were kept in 10% formol saline for histopathological processing.

Biochemical Studies

Lipid peroxidation

Lipid peroxidation was determined in liver tissue homogenates by measurement of malondialdehyde (MDA) by the method of Ruiz-Larrea et al., (1994). In this assay, thiobarbituric acid reactive substances (TBA) react with thiobarbituric acid to form TBA-MDA adduct which can be measured using spectrophotometer at 532 nm.

Reduced Glutathione

Reduced glutathione was measured in liver tissue homogenates using the procedure of Ellman et al., (1959). The assay is based on the reduction of Ellman´s reagent (DTNB; 5, 5′-dithiobis (2-nitrobenzoic acid)) by the free sulfhydryl group on GSH to produce a yellow colored 5-thio-2-nitrobenzoic acid which can be measured using spectrophotometer at 412 nm.

Hydroxyproline

Liver tissue content of hydroxyproline content was determined with the colorimetric method described by Bergman and Loxley, (1963).

Histopathological and Histochemical Studies

Five μm thick paraffin sections were cut and stained with Hematoxylin & Eosin (HX & E) for the histopathological study. Periodic acid Schiff's (PAS) stain was used for the detection of mucopolysaccharides in liver. Masson`s trichrome stain was used for detecting collagen deposition.

Cleaved Caspase-3 Immunostaining

Mouse monoclonal caspase-3 antibodies were used for the detection of cleaved caspase-3 in liver. Briefly, paraffin sections were heated in a microwave oven for 25 min at 720 W for antigen retrieval and then incubated with anti-caspase antibody overnight at 4°C. After washing with PBS, followed by incubation with biotinylated goat-anti-rabbit-immunoglobulin G secondary antibodies (1:200 dilution: Dako Corp.) and streptavidin/alkaline phosphatase complex (1:200 dilution: Dako) for 30 min at room temperature, the binding sites of antibody were visualized with DAB (Sigma, USA). After washing with PBS, the samples were counterstained with H & E for 2–3 min, dehydrated using graded alcohols and xylene and mounted on slides. The immune staining intensity and cellular localization of cleaved caspase-3 were analyzed by light microscope.

Quantitative Histochemical and Immunohistochemical Studies

Quantitative measurements of mucopolysaccharides, fibrosis and cleaved caspase-3 in the liver were done with the use of computerized image analyzer (Leica Qwin 500 image) in the Image Analyzer Unit, Pathology Department, NRC. Ten fields were chosen for each slide and the mean values were obtained.

Statistical Analyses

Results in the study are expressed as mean ± SE. Statistical analysis of data was done using one way analysis of variance (ANOVA) followed by Tukey's multiple comparisons test. Graph Pad Prism 6 software for Windows was used (Graph Pad Prism Software Inc., San Diego, CA, USA). Statistical significance was considered at a probability value $p < 0.05$.

3. RESULTS

Biochemical Results

Results are presented in table 1 & figure 1. Compared with the vehicle control group, the administration of 2,4-DNP resulted in significant decrease in malondialdehyde and reduced glutathione contents in liver. Meanwhile, significant increase in liver tissue hydroxyproline content was observed after high doses of 2,4-DNP. These effects of 2,4-DNP were dose-dependent.

Table 1 Effect of different doses of 2,4 dinitrophenol (2,4-DNP) on liver malondialdehyde (MDA), reduced glutathione (GSH) and hydroxyproline contents.

Groups/ Parameters	Control	2,4-DNP 10 mg/kg	2,4-DNP 40 mg/kg	2,4-DNP 80 mg/kg	2,4-DNP 160 mg/kg
MDA (nmol/g. tissue)	13.08 ± 0.21	11.8 ± 0.88 (-9.8%)	9.01 ± 0.99* (-31.1%)	5.68 ± 0.06*+ (-56.6%)	5.0 ± 0.11*+ (-61.8%)
GSH (μmol /g.tissue)	7.62 ± 0.46	7.49 ± 0.16 (-1.7%)	6.81 ± 0.09 (-10.6%)	5.99 ± 0.09* (-21.4%)	4.01 ± 0.04*+ (-47.4%)
Hydroxyproline content (μg/g. protein)	153 ± 11.8	159 ± 9.8 (3.9%)	170.8 ± 5.8 (11.6%)	188.1± 6.9* (22.9%)	215.8 ± 7.6*+ (41.0%)

Data are expressed as mean ± SE. The percent change from the control value is shown in parenthesis. Statistical analysis was performed with the use of one-way ANOVA. *: $p < 0.05$ vs. control. +: $p < 0.05$ *vs.* 2,4-DNP 10 mg/kg.

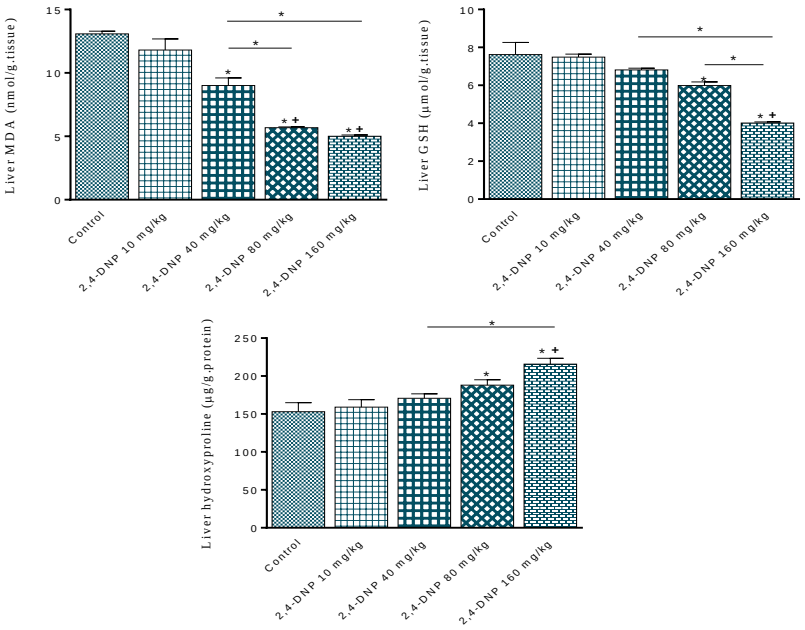

Figure 1 Effect of different doses of 2,4 dinitrophenol (2,4-DNP) on liver malondialdehyde (MDA), reduced glutathione (GSH) and hydroxyproline. *: $p < 0.05$ vs. control and between different groups as indicated in the graph. +: $p < 0.05$ *vs.* 2,4-DNP 10 mg/kg.

Histopathological Results

Sections of the control rat liver showed normal histological architecture. The liver appeared to be composed of ill-defined hexagonal classic lobules, the hepatic lobules arranged in cords radiating from the central veins (cv) appeared to be made of hepatocytes (Figure 2A). Rats treated with 2,4-DNP 10 mg/kg revealed dilatation and congestion of the portal vein with thickened wall, aggregates of inflammatory cell infiltration and congested blood sinusoid. Some hepatocytes appeared with pyknotic nuclei (Figure 2B & C). In 2,4-DNP 40 mg/kg-treated group, massive degenerative changes in the form of hepatocytes with pyknotic nuclei, dilatation and congestion of portal vein, inflammatory cell infiltrate and fibrosis around the portal tract were demonstrated. Dilated and congested blood sinusoids were clearly observed (Figure 2D). Rats treated with 2,4-DNP 80 mg/kg showed distortion of hepatic architecture, dilatation and congestion of portal vein and dilated bile ducts, massive aggregates of inflammatory cell infiltrate and hypertrophy and hyperplasia of Kupffer cells (Figures 2E & F). Severe histopathological changes were demonstrated in the liver of rats treated with 2,4-DNP 160 mg/kg in the form focal areas of coagulation necrosis with reddish color of hemorrhage, dilated and congested portal vein and blood sinusoids. Cellular infiltration could be observed. Hepatocytes showed deeply stained pyknotic nuclei (Figure 2G, H & I).

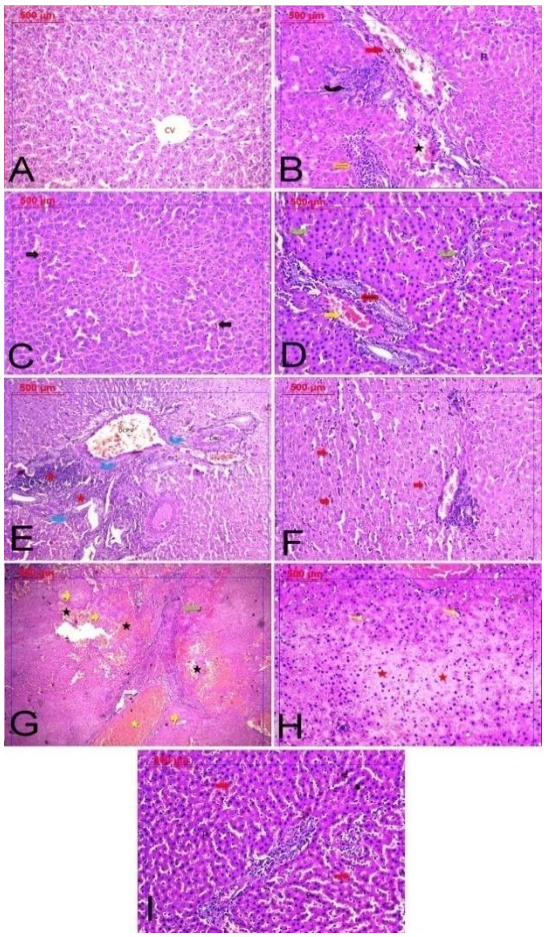

Figure 2 Photomicrographs of Hx & E stained sections of the liver. (A) Control: normal hepatic architecture. (B) 2,4-DNP 10 mg/kg: dilatation and congestion of the portal vein (DCPV), dilated bile duct (star) and thickening of wall (red arrow). Inflammatory infiltrate (orange arrow), hepatocytes with pyknotic nuclei (P). (C) 2,4-DNP 10 mg/kg: dilated and congested blood sinusoid (black arrow). (D) 2,4-DNP 40 mg/kg: most hepatocytes appeared with pyknotic nuclei (P) dilatation and congestion of portal vein (yellow arrow), inflammatory infiltrate (red arrow) and fibrosis (white arrow). Dilated and congested blood sinusoids (light green arrow). (E) 2,4-DNP 80 mg/kg: distortion of hepatic architecture, dilatation and congestion of portal vein (DCPV), dilated bile duct (DBD). Massive aggregate of inflammatory cell infiltrate (star) and fibrosis (light blue arrow). (F) 2,4-DNP 80 mg/kg: hypertrophy and hyperplasia of Kupffer cells. (G) 2,4-DNP 160 mg/kg: coagulative necrosis (yellow arrow) with reddish color of hemorrhage (black star). Dilated congested portal vein (yellow star), fibrosis (light green arrow). (H) 2,4-DNP 160 mg/kg: coagulative necrosis (star), minute vacuolar degeneration. (I) 2,4-DNP 160 mg/kg: deeply stained pyknotic nuclei (P) in hepatocytes. Dilated, congested blood sinusoid (red arrow) and inflammatory cells (white arrow) (Hx & E x200).

Periodic Acid Schiff's Staining

The glycogen in liver tissue stained by Periodic acid Schiff's (PAS) in the control rats deeply stained reddish granules in the cytoplasm of hepatocytes. The peripheral zonal cells (Zone III; plays the largest role in glycogen synthesis) showed more glycogen granules than the central cells. Rats treated with 2,4-DNP 10, 40, 80, and 160 mg/kg exhibited increase in PAS reactivity in dose-dependent manner (Figure 3). Figure 4 shows the morphometric measurements for PAS area % in liver.

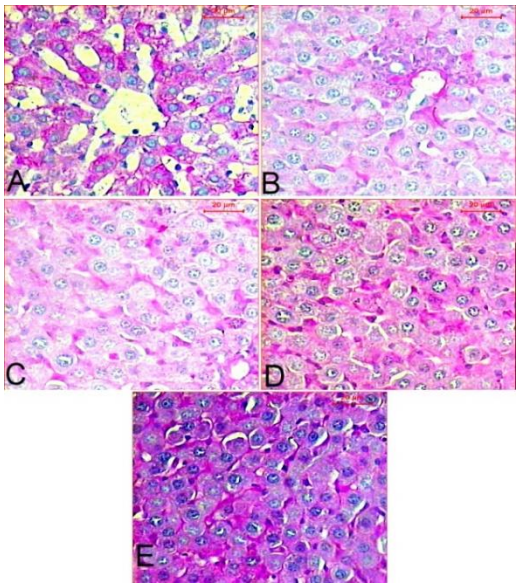

Figure 3 Photomicrographs of liver sections stained with Periodic acid Schiff's (PAS): Positive reaction in the cytoplasm of hepatocytes. (A) Control. (B) 2,4-DNP 10 mg/kg: Slight increase in PAS reactivity**.** (C & D & E): 2,4-DNP 40, 80, 160 mg/kg: Apparent increase in the PAS reactivity (PAS reaction X 400).

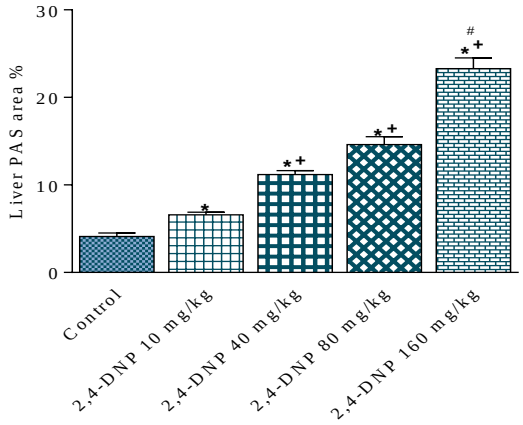

Figure 4 Results of morphometric measurements of PAS area% for liver tissue in different groups. Data are expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA. *: $p < 0.05$ *vs.* control. +: $p < 0.05$ *vs.* 2,4-DNP 10 mg/kg. #: $p < 0.05$ *vs.* 2,4-DNP 40 mg/kg.

Masson's Trichrome Staining

Sections stained with Masson's tri chrome stains from the control rats showed the collagen is disposed as wavy fibrils either singly or fused together in dense bundles (Figure 5A). Mild increase of collagenous fibers around the portal tract was seen in rats treated with 2,4-DNP 10 mg/kg (Figure 5B). Moderate increase in area of fibrosis were also observed after 2,4-DNP 40 or 80 mg/kg (Figure 5 C & D). Marked increase in area of fibrosis around the portal tract was seen in liver sections from rats treated with 2,4-DNP 160 mg/kg (Figure 5E). Figure 6 shows the morphometric measurements for fibrosis area %.

Caspase-3 Immunoreactivity

No caspase-3 immunoreactivity was detected in the control rats (Figure 7A). Morphometric analysis revealed moderate expression in area percentage of caspase-3 immmunoreactivity in rats treated 2,4-DNP 10 or 40 mg/kg (Figure 7B & C). The expression of caspase 3 was increased with 2,4-DNP 80 or 160 mg/kg (Figure 7D & E). The morphometric measurements of caspase-3 area % in liver are shown in figure 8.

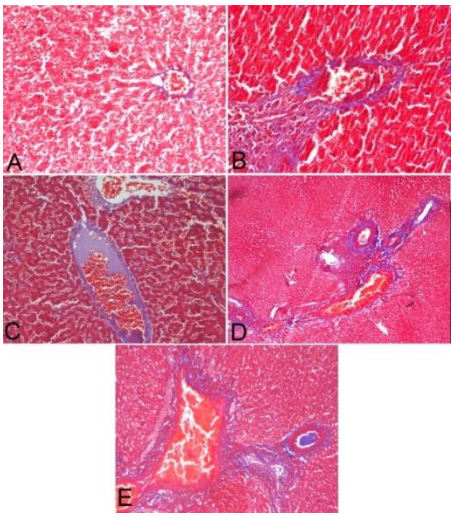

Figure 5 Photomicrographs of liver sections stained with Masson tri chrome stain showing collagen disposed as wavy fibrils either singly or fused together in dense bundles. (A) Control. (B) 2,4-DNP 10 mg/kg: showing mild deposition of fibrous tissue. (C & D): 2,4-DNP 40, 80 mg/kg: Moderate deposition of fibrous tissues. (E) 2,4-DNP 160 mg/kg: Showing marked deposition of fibrous tissue (Masson tri chrome stain X 200).

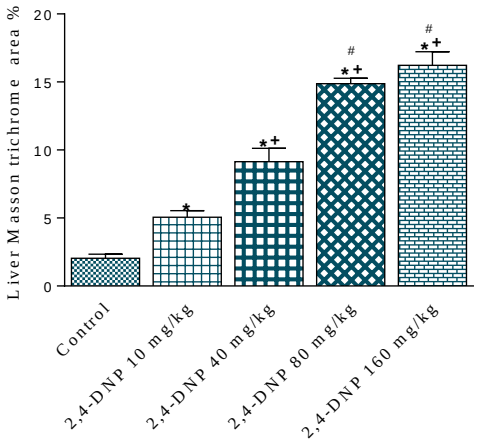

Figure 6 Results of morphometric measurements of Masson tri chrome area % in different groups. Data are expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA. *: $p < 0.05$ *vs.* control. +: $p < 0.05$ *vs.* 2,4-DNP 10 mg/kg. #: $p < 0.05$ *vs.* 2,4-DNP 40 mg/kg.

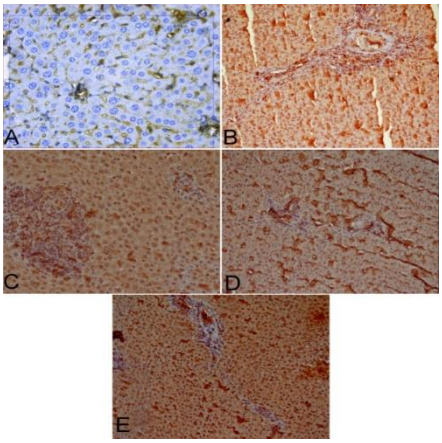

Figure 7 Photomicrographs of immune stained liver sections for cleaved caspase-3 showing: (A) No expression of caspase-3 in the control group. (B & C) moderate expression in cleaved caspase-3 in rats treated with 2,4-DNP at 10 and 40 mg/kg. (D & E) Increased expression in rats treated with 2,4-DNP at doses of 80 and 160 mg/kg, respectively (X200).

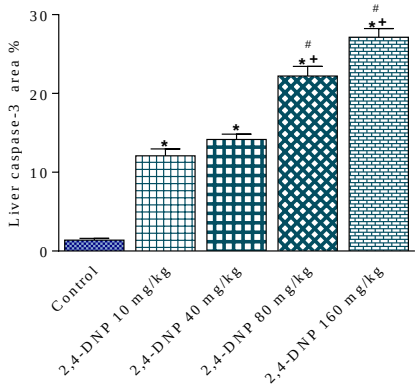

Figure 8 Results of morphometric measurements of caspase-3 area % in liver. Data are expressed as mean ± SE. Statistical analysis was carried out by one-way ANOVA. *: $p < 0.05$ *vs.* control. +: $p < 0.05$ *vs.* 2,4-DNP 10 mg/kg. #: $p < 0.05$ *vs.* 2,4-DNP 40 mg/kg.

4. DISCUSSION

The results of this study clearly indicated that the slimming agent 2,4 DNP produced severe degenerative changes in the liver form distortion of hepatic architecture, dilatation and congestion of portal vein, massive aggregates of inflammatory infiltrate and fibrosis, progressing to focal areas of coagulation necrosis as the dose of 2,4 DNP is increased. The results of this work are in agreement with Poole and Haining, (1934) who demonstrated the degeneration of hepatocytes in the periphery of the lobules, granular cytoplasm and pyknotic nuclei in periportal cells in the liver of a woman who took 7 mg/kg/day 2,4-DNP for 5 days. In addition, the United States Environmental Protection Agency, (2007) stated that microscopic examination of rats treated with 2-4 DNP showed slight congestion and cloudy swelling of the liver. Necrosis of hepatocytes and hemorrhage in the liver were reported in a woman who took undetermined dose of 2,4-DNP for 1 week, whereas severe fatty changes were found in the liver of a young girl who took 1.0 mg/kg/day of 2-4 DNP (1936). According to Lu et al., (2011) hepatic injury and neutropenia might be related to direct toxic effects or systemic inflammatory response syndrome induced by 2,4-DNP. The several derivatives of 2,4-DNP would be preferentially metabolized by the cytochrome P450 system in the liver to the active protonophore DNP. The high concentrations of 2,4-DNP and other nitrophenols are toxic to humans and animals. Toxicity is caused by interference with cellular energy metabolism due to uncoupling of oxidative phosphorylation (Ferreira and De Felice, 2007).

We used Periodic acid Schiff (PAS) for delineating the effect of 2,4-DNP on the cell's glycogen content in liver. PAS reaction increased in hepatocytes. An increase in hepatic glycogen infiltration was also reported in rats treated with DNP (Evers et al., 2015). Similarly, Perry et al., (2015) found that the treatment of obese rats with DNP induced an 80% increase in glycogen content. The use of Masson's tri chrome stain in our study also indicated an increase in fibrosis in the liver tissue of rats treated with 2,4-DNP at different doses. Hepatic fibrosis occurs in response to liver damage and regenerated apoptotic cells after repeated injury (Friedman et al., 2002). The inflammatory response is accompanied by limited deposition of extra cellular matrix (ECM), so that if the regeneration of dying cells fails during persistent liver injury, hepatocytes are replaced by abundant ECM, including fibrillar collagen, depending on the origin of injury (O'Connell and Rushworth, 2008). In this process, hepatic stellate cells acquire a highly proliferative index producing fibrillar collagen within the injured liver (Salama et al., 2013).

Caspases are cysteine-aspartic proteases, essentially involved in apoptotic cell death and inflammation. In apoptosis or programmed cell death there is a controlled dismantling of intracellular components without inducing inflammation or damage to neighboring cells. The morphological hallmarks being DNA fragmentation and membrane blebbing. Caspase-3 is an executioner caspase and an essential component in some apoptosis pathways (McIlwain et al., 2013). In the present work we showed that cleaved caspase -3 immunostaining increases in liver tissue of 2,4-DNP-treated rats. In their study, Mohamed and Magdy, (2017) found that the increase in caspase-3 was most evident as diffused nuclear and cytoplasmic positivity in the apoptotic hepatocytes. This is due to activation of caspase-3 in apoptotic liver cells by the hypoxia (Ozaki et al., 2012).

It has been suggested that the ability of the drug/chemical to cause cell apoptosis or necrosis will depend on the extent of functional impairment of the mitochondria. It is likely that in presence of an uncoupler of oxidative phosphorylation like 2,4-DNP, the deficiency of ATP will be a major determinant of the fate of the cell i.e., by apoptosis or necrosis. The latter being the case with profound mitochondrial dysfunction and ATP depletion (Grattagliano et al., 2002). Our results showed that the deleterious effects for 2,4-DNP on the liver was not due to oxidative stress as evidenced by the marked decrease in the end product of lipid peroxidation, malondialdehyde (Gutteridge, 1995). There was also depletion of reduced glutathione, reflecting in this case

decreased synthesis as a result of reduction in ATP synthesis. It has been suggested that depression of cell energy impairs cellular functions, ability to control the intracellular Ca^{2+} homeostasis and the cell's redox state. With limited decrease in ATP production, there is activation of the apoptotic cell death program by release of cytochrome c but severe energy failure will ultimately leads to cell necrosis (Seppet et al., 2009).

5. CONCLUSIONS

In summary, the present study indicates that the administration of high doses of 2,4-DNP was associated with histologic liver damage and apoptosis. 2,4-DNP resulted in inhibition of lipid peroxidation and depletion of reduced glutathione. These deleterious effects of 2,4-DNP is accounted for by uncoupling of oxidative phosphorylation and the consequent energy failure.

Author contribution

O.M.E.A.S. and F.A.M designed and conducted the research. D.M performed the biochemical studies. F.A.M, M.E.S and N.N.Y performed the histopathological studies and its interpretation. O.M.E.A.S. and F.A.M wrote and prepared the manuscript. O.M.E.A.S., F.A.M, M.E.S, N.N.Y and D.M. approved the final version of the manuscript.

Ethical approval

Animal procedures followed the guidelines of the Institute ethics committee for the use of animals in experimental studies and the Guide for Care and Use of Laboratory Animals by the U.S. National Institutes of Health (Publication No. 85-23, revised 1996).

Informed consent

Not applicable.

Conflicts of interests

The authors declare that there are no conflicts of interests.

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The study has not received any external funding.

Data and materials availability

All data associated with this study are present in the paper.

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